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Monitoring of the impact of *Prestige* oil spill on *Mytilus galloprovincialis* from Galician coast

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Abstract

In November 2002, the *Prestige* oil tanker was wrecked in front of Galician coast (NW of Spain), spilling near 63,000 tons of heavy oil until February 2003. Contamination produced was very extensive (70% of Galician beaches were reached by the oil) but heterogeneous, alternating intensely affected zones with neighbour locations where the repercussion was minimal. The objective of this study was to monitor sea environment contamination caused by *Prestige* oil spill during an 11-month period (August 2003–June 2004, nine samplings) in two locations of Galician coast with different geographical properties (Lira and Ancoradoiro beaches), by means of chemical determination of total polycyclic aromatic hydrocarbons (TPAH) in seawater, and using as exposure biomarker TPAH content in mussel (*Mytilus galloprovincialis*) tissues, and as effect biomarker DNA damage in mussel gills, evaluated by the comet assay. In addition, recovery ability of the mussels was determined after a 7-day stay in the laboratory. TPAH contents in seawater were very high in the earliest samplings, but then they maintained below 200 ng L^{-1}, similar to reference seawater. However, TPAH levels in mussel tissues were more variable: they increased again from January 2004, probably due to the adverse meteorological conditions that turned over the sea bottom and dispersed the oil accumulated in sediments. In most samplings, these levels decreased during the recovery stage. DNA damage in oil-exposed mussels was significantly higher than in reference mussels, both before and after the recovery phase, but they did not differ to one another. Comet tail length was slightly reduced during the recovery stage, indicative of a certain DNA repair in exposed mussels. This study showed up the importance of monitoring sea contamination events during an extended time, not only in evaluating the presence of the contaminants in the environment but also in determining their bioaccumulation and their effect on the exposed organisms.
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Keywords: *Mytilus galloprovincialis*; *Prestige*; PAH; Comet assay; Galician coast

1. Introduction

The accident of the oil tanker *Prestige* in front of Galician coast (NW of Spain) in November 2002 represented one of the largest environmental catastrophes occurred in the European navigation. Between 40,000 and 60,000 merchant ships pass in front of Galician coast each year (approximately 120 per day), 20% of them carrying dangerous products (González-Laxe, 2004). The intense traffic is focused in a two-way sea highway, that maintains the space between ships and move them further away 20–30 miles from the coastline. Although the traffic of the ships is controlled, the hardness of the sea, mainly during winter storms, and the danger of this coast make Galicia the European region with the greatest number of this kind of disasters. Some examples of tankers that sank in this coast in the last decades are *Polycommander* (1970), *Erkowit* (1973), *Urquiola* (1976), *Andros Patria* (1979) or *Aegean Sea* (1992).

The breakage of the *Prestige* tanker hull and its sinking at a depth of 4000 m caused the spill of 63,000 tons of heavy oil until February 2003 that generated four black tides

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and the arrival of lots of oil to the shore. According to the report presented by the Spanish Government to the International Oil Pollution Compensation Funds, 70% of the Galician beaches were affected by the oil (IOPC Funds, 2003). Contamination produced by *Prestige* spill was very extensive but heterogeneous due to the special geographical characteristics of Galician coast and to the meteorological conditions and sea currents. Thus, intensely affected zones alternated with neighbour locations where the repercussion was minimal.

In general, spilled oil experiments a slow degradation, in which several factors like oxygen and sunlight take part. Its chemical composition changes by means of a process called "aging". First, low molecular weight compounds are lost by evaporation, dissolution and degradation. Heavier compounds disperse as drops that can stay for a long time, and may be swept out by the currents. The remaining crude undergoes photooxidation; part of it dissolves in seawater, and the other part forms a gelatinous emulsion w/o that turns into dense semisolid tar balls with asphaltic appearance. The most important properties that affect the behaviour of an oil spilled to the sea are its volatility, solubility, tendency to emulsion and degradation capacity. *Prestige* oil presented low solubility, high viscosity and adherence, low dispersion capacity and slow degradation. Thus, it was a highly persistent oil both in seawater and adhered to surfaces.

Prestige oil has been classified as fuel oil No. 6, containing 2.85% sulphur. The International Agency for Research on Cancer has regarded this kind of oil as possible human carcinogen (group 2B) (IARC, 1989). The analyses made by the Spanish National Research Council (CSIC) revealed 50% aromatic hydrocarbons, 22% saturated hydrocarbons and 28% resins and asphalthene (CSIC, 2003). Thus, it was a relatively low volatile oil (5–10%) due to its greater content in high molecular weight hydrocarbons, such as polycyclic aromatic hydrocarbons (PAH). Six of the PAH contained in *Prestige* oil (naphthalene, benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene and dibenz[*ah*]anthracene) have been catalogued by the IARC as possible or probable human carcinogens (groups 2A and 2B), and are part of the 16 PAH designated by the United States Environmental Protection Agency (USEPA) as primary contaminants. In addition, *Prestige* oil contained several metals, such as Cu, Cd and Pb (Prego and Cobelo-García, 2004).

Marine organisms can take up contaminants from bottom sediments, suspended particulate material, the water-column and food-sources (Varanasi et al., 1992; Livingstone, 1993). In corporation rate will depend not only on the availability of the contaminants but also on several biotic and abiotic factors. In this context, biomonitoring constitutes a good alternative or complement to chemical systems for contamination evaluation. Mussels (genus *Mytilus*) result very suitable for genotoxic biomonitoring of the sea environment because of their abundance, their suitable size, their wide distribution in both hemispheres, and their filter feeding activity that favours the bioaccumulation of contaminants (Khadim, 1990). In fact, they are considered as good sentinel organisms. Exposure of aquatic organisms such as mussels to genotoxic contaminants could pose a risk to human health via the food chain, as has been reported by Lemiere et al. (2005), who described genotoxic effects in rats fed with mussels contaminated by the *Erika* oil spill. Nevertheless, there could be also an ecological risk that may lead to heritable mutations and loss in the total genetic diversity (either intra- or inter-species) with significant implications for long-term survival of natural populations (Bikham et al., 2000).

The consequences of exposure and metabolism of chemicals may be assessed by investigating biomarkers of contaminant damage, for example, DNA strand breaks (Mitchelmore et al., 1998). Breaks may be directly produced by chemicals or may result from alkali-labile sites or during excision repair of DNA (Fairbairn et al., 1995). Techniques commonly used to detect strand breaks in aquatic organisms are alkaline elution (Bihari et al., 1992) or alkaline unwinding (Nacci et al., 1992; Everaarts, 1995; Liepelt et al., 1995), but the comet assay is increasingly used because it appears as a sensitive, rapid and economic technique for the detection of DNA strand breaks, which has many attributes as a non-specific biomarker of genotoxicity in aquatic invertebrates (Livingstone et al., 2000; Lee and Steinert, 2003).

The objective of this study was to monitor sea environment contamination caused by *Prestige* oil spill during an 11-month period in two locations of Galician coast with different geographical properties in terms of their coastline shape and swell conditions, by means of chemical determinations of PAH in seawater and using as exposure biomarker PAH content in mussel (*Mytilus galloprovincialis*) tissues, and as effect biomarker DNA damage in mussel gills, evaluated by the comet assay. In addition, recovery ability of the mussels was determined after a 7-day stay in the laboratory.

2. Materials and methods

2.1. Sample collection

Mussels (*M. galloprovincialis*) 6–8 cm in length were collected from natural populations located at Lira and Ancoradoiro beaches (UTM coordinates 490285.01 North and 4735989.19 East for Lira; 491107.77 North and 4733812.01 East for Ancoradoiro, time zone 29, North Hemisphere), both belonging to Carnota town (Figs. 1 and 2). These beaches were chosen because they were intensely affected by *Prestige* oil spill and have different coastline shape and exposure to swell and marine currents. Reference mussels were collected from Perbes beach (UTM coordinates 564057.95 North and 4802631.84 East, time zone 29, North Hemisphere), not

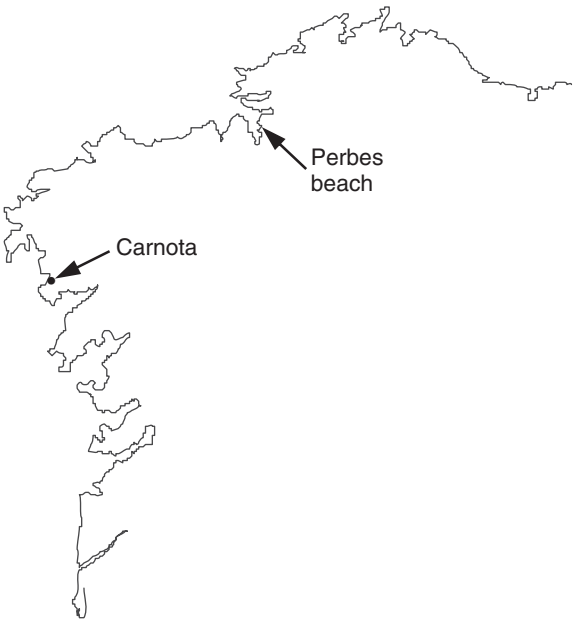


Fig. 1. Map of Galician coast showing location of Carnota town (sampling site of *Prestige* oil-affected mussels) and Perbes beach (sampling site of reference mussels).

affected by the oil spill because of its sheltered location inside Betanzos estuary. Together with mussels, seawater samples (1 L) were taken in glass bottles. Samples were transported immediately to the laboratory (less than 2 h), and mussels were placed in filtered seawater (total PAH content <5 ng L^{-1}) in a photoperiod chamber at 18 °C in cycles of 12-h light–darkness for 1 day, to clear gut contents prior to the experiments. For each location, 20 g mussel tissues (4–6 individuals) were used for PAH content determination, and 9 individuals were randomly selected for DNA damage evaluation by means of comet assay.

Remaining mussels were maintained in laboratory conditions for 7 days more (recovery period). They were daily fed with two species of microalgae (*Isochrysis galbana* and *Tetraselmis suecica*) and feeding was interrupted 24 h before end of the experiment. Then new samples were taken to determine PAH content and DNA damage.

Nine samplings were carried out in 30–50 days intervals, for an 11-month period. Dates of the samplings were: August 2003, September 2003, October 2003, November 2003, January 2004, February 2004, March 2004, April 2004 and June 2004.

2.2. Determination of PAH content

Seawater samples were prepared for the analysis following USEPA protocol (2000). Mussel tissue samples were prepared as described in Baumard et al. (1999). Determinations of PAH content were performed in triplicate by means of a gas chromatography–mass spectrometry methodology (GC–MS) (Baumard et al., 1999). The PAH determined were: naphthalene, 2-methylnaphthalene, biphenyle, 2,6-dimethylnaphthalene, 1,2-dimethylnaphthalene, acenaphthylene, acenaphthene, 2,3,5-trimethylnaphthalene, fluorene, 1-methylfluorene, dibenzothiophene, phenanthrene, anthracene, 2-methylanthracene, 1-methyllphenanthrene, fluoranthene, pyrene, 11*H*-benzofluorene, 1,2-benzodiphenylensulfure, benz[*a*]anthracene, triphenylene, chrysene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, benzo[*ghi*]perylene, dibenz[*ah*]anthracene, indopyrene, 2-methyldibenzothiophene, 2,4-dimethyldibenzothiophene, 2-propylbenzothiophene and 2-butylbenzothiophene. The index total PAH (TPAH) was calculated as the sum of the total quantity of all these compounds.

2.3. Comet assay

Scissors and tweezers were used to excise and dissect mussel gills. They were placed in a tube containing 8 mL of calcium magnesium-free saline solution (CMFS: 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl and 5 mM EDTA) and left for 1 h in soft horizontal shaking. Tubes were then placed in an ice container in vertical for 5 min, to allow the fragments of tissue to settle. The supernatant containing suspended cells was collected with a pipette and centrifuged at 1500 rpm for 5 min at 4 °C. The pellet was resuspended in 500 μL of a solution containing 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$, and Trypan blue exclusion technique was applied to test cell viability.

The alkaline comet assay was performed following Wilson et al. (1998), with minor modifications (Pérez-Cadahía et al., 2004). One hundred randomly selected cells from each individual (50 per replicate slide) were examined by a "blind" scorer, using a magnification of 400×. Image capture and analysis were performed using the QWIN Comet software (Leica Imaging Systems, Cambridge, UK). Comet tail length (TL), measured from the estimated centre of the cell, was used to evaluate DNA damage.

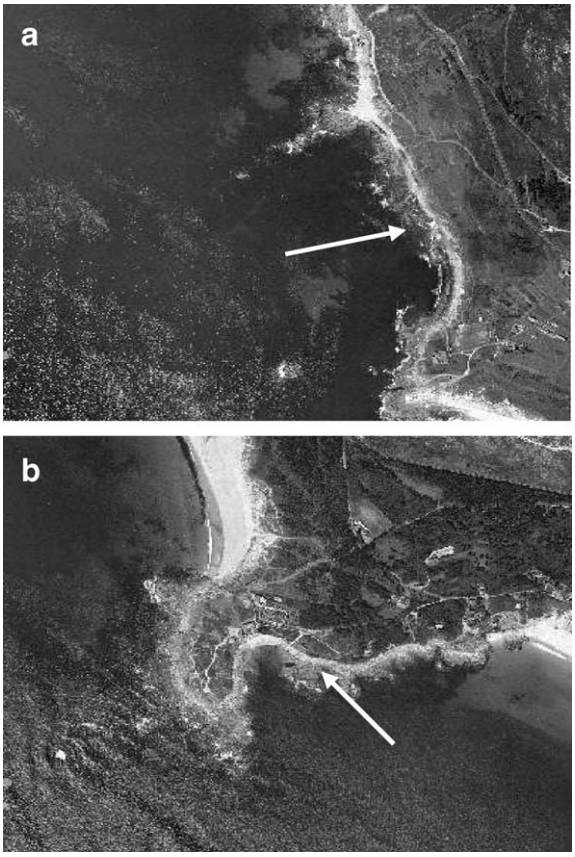


Fig. 2. Aerial views of Lira (a) and Ancoradoiro (b) beaches. Arrows point to the mussels sampling sites.

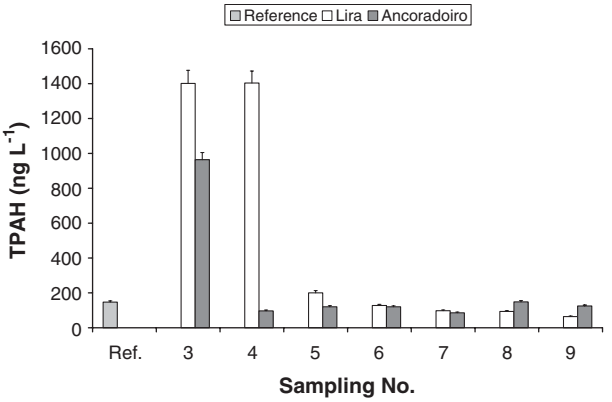


Fig. 3. TPAH contents in seawater samples from the reference site (Perbes beach), and from Lira and Ancoradoiro beaches. Ref.: reference seawater.

2.4. Statistical analyses

Statistical analyses were performed using the SPSS for Windows statistical package, version 11.0 (IL, USA). Kolmogorov–Smirnov goodness-of-fit test was applied to compare the distribution of the variables obtained in the study with the normal distribution. As the distribution of all variables departed significantly from normality, non-parametric tests were deemed adequate for the statistical analyses of these data. The existence of significant differences among groups were determined by means of Kruskal–Wallis test, followed by Mann–Whitney *U*-test, to evaluate the effect of origin, sampling time, and recovery period. Associations between two variables were analysed by Spearman's correlation. Level of significance was set at 0.01.

3. Results

PAH content in seawater samples and mussel tissue samples were both analysed by means of a GC–MS technique, and results obtained are gathered in Figs. 3 and 4, respectively. Analyses of seawater samples from first and second samplings could not be performed because of storage problems. Mean TPAH value obtained in the reference water was 146.99±6 ng L^{-1}. TPAH contents were very high in samplings 3 and 4 from Lira, and in sampling 3 from Ancoradoiro, but they maintained low and rather constant from sampling 5. In general, TPAH was higher in samples from Lira in the earliest samplings, and lower in the latest samplings.

Table 1
Results of comet assay (mean values of all samplings)

Origin	TL (μm, mean±S.E.)		
	Total	Arrival	Recovery
Reference	18.94±0.18	18.78±0.17	19.11±0.12
Lira	22.96±0.12[a]	23.67±0.16[a]	22.05±0.17[a,b]
Ancoradoiro	23.65±0.11[a]	24.32±0.16[a]	22.90±0.15[a,b]

[a] $P<0.01$, significant difference with regard to reference.
[b] $P<0.01$, significant difference with regard to arrival.

Mean TPAH value obtained in reference mussels was slightly lower at the arrival than after the recovery stage, although both of them maintained below 200 μg kg^{-1}. Levels of TPAH in mussel tissues from both locations were highly variable. As in seawater samples, analyses of tissues at the arrival reflected higher TPAH values in samples from Lira in the earliest samplings, and on the contrary in the latest samplings. Generally, mussels reduced their values of TPAH contents during the 7-day recovery phase, but there were some exceptions such as samplings 6, 7 and 9 from Lira and samplings 5 and 7 from Ancoradoiro.

Table 1 shows results obtained in comet assay carried out with mussel gills from the two locations and the reference site. Comet tail length values obtained in the samples from Lira and Ancoradoiro were significantly higher than those obtained for reference mussels, both before and after the recovery time. In addition, DNA damage in mussels from the two contaminated locations studied was significantly reduced after the recovery phase. Details of tail length values obtained for each separate sampling are reflected in Fig. 5. Tail length was usually lower after the recovery time than before, although there were some samplings presenting higher DNA damage after the 7-day stay in the laboratory, as samplings 3, 4, 7 and 8 from Lira and samplings 4, 5 and 9 from Ancoradoiro. Significant differences between tail length at the arrival and after the recovery time

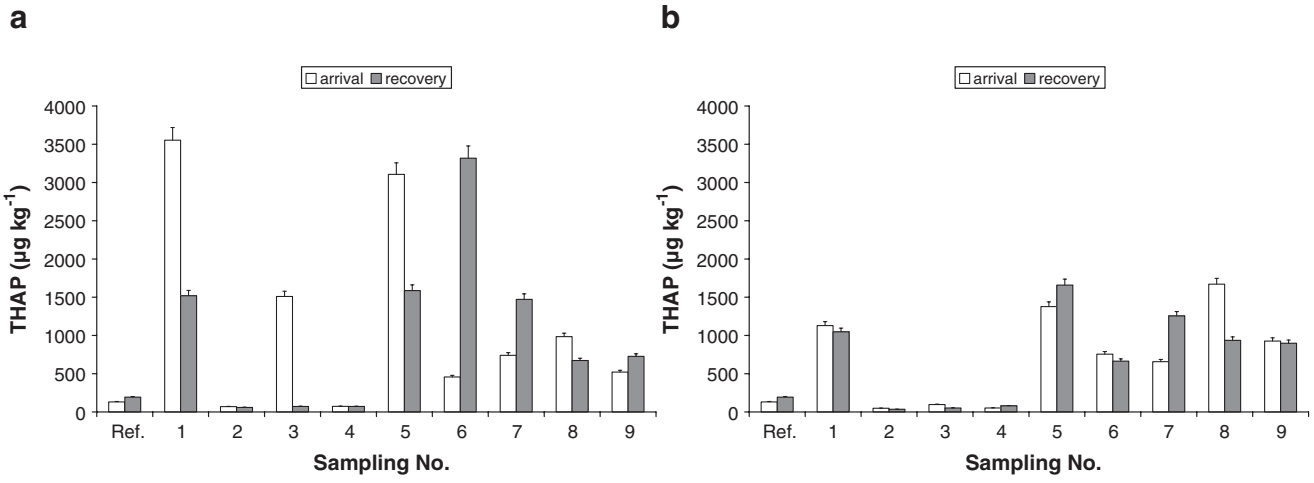


Fig. 4. TPAH contents in mussel tissues from Lira (a) and Ancoradoiro (b), before and after the recovery stage. Ref.: reference mussels.

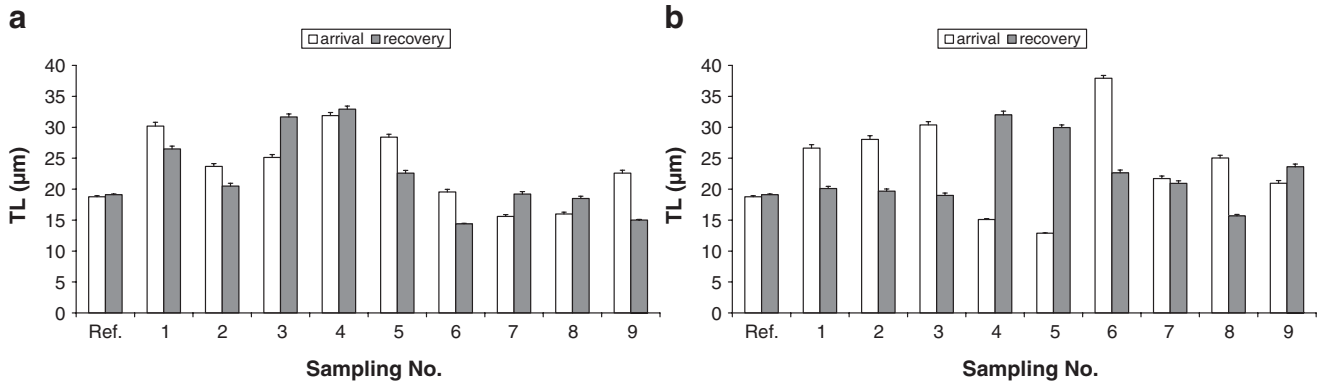


Fig. 5. DNA damage in mussels from Lira (a) and Ancoradoiro (b), before and after the recovery stage. Ref.: reference mussels.

were obtained in all cases, except in sampling 7 from Ancoradoiro.

Associations between variables obtained in this study (TPAH contents in seawater and in mussel tissues, and TL) were analysed, obtaining good correlation coefficients for TPAH content in seawater and TL, both in Lira ($r=0.714$, $P=0.071$) and Ancoradoiro ($r=0.750$, $P=0.056$) and in the total data ($r=0.696$, $P<0.01$).

4. Discussion

Nowadays, oil spills represent one of the main causes of marine contamination, caused fundamentally by accidents of oil tankers either near to the coast or far away from it. The environmental impact of these spills affects not only to the species biodiversity whose natural environment results seriously damaged but also to human populations whose marine economic resources are decreased.

In this work, the extent and impact of contamination caused by *Prestige* oil spill has been evaluated by monitoring during an 11-month period the PAH concentration in the seawater, the bioaccumulation of PAH in a sentinel organism (the mussel *M. galloprovincialis*), and the effect induced determined as DNA damage (comet assay) in mussel gills. This monitoring has been performed in two locations of the Galician coast with different geographical conditions intensely affected by the spill, and compared to a reference site not reached by the spill because of its sheltered location inside an estuary. Mussels have been effectively used as bioindicator organisms in other marine catastrophes, such as in a study carried out after *Sea Empress* spill in Wales in 1996 (Dyrynda et al., 1997).

TPAH contents in seawater were very high in the earliest samplings, but then they maintained below 200 ng L^{-1}, level considered as medium by some authors (Hellou et al., 2005) and similar to the concentration obtained in the seawater from the reference site (146.99±6 ng L^{-1}). As time went by TPAH levels in seawater decreased. This could probably be caused by the loss of some oil components by degradation or by volatilization of lighter compounds due to an "aging" process. In addition, sedimentation has been shown to be an important mechanism for removal of heavy oil from the water column following some spills (Wolfe, 1977).

In aquatic environments PAH toxicity increases with molecular weight, and their bioaccumulation is prone to be fast (Eisler, 1987). The main impacts of PAH are generated by their genotoxic properties, showing a great part of them carcinogenic activity and being included in groups 1, 2A and 2B of IARC. TPAH concentrations in mussel tissues were more variable. After an initial decrease, hydrocarbon content increased again from sampling 5 (January 2004) in mussels from both Lira and Ancoradoiro. One explanation could be that the adverse meteorological conditions at that winter time turned over the sea bottom and dispersed the oil accumulated in sediments, or that the swell removed oil stains on the rocks and the oil passed again to the seawater being at the disposal of the mussels (either in solution or adhered to phytoplankton particles) that incorporate it while filtering seawater for feeding. In fact, it has been reported that PAH content in sediments from Galician coast increased from December 2002 to September 2003, a usual event in the temporal evolution of oil spills as a consequence of sedimentation of hydrocarbons in suspension (IEO, 2003). In addition, mussels filter ability depends on the reproductive cycle of the animals (Hellou et al., 2005).

Differences observed in TPAH contents in mussel tissues between Lira and Ancoradoiro are probably due to their geographic location (Fig. 2). Lira beach is completely exposed to the beat of the Atlantic sea, with strong swell conditions. On the contrary, Ancoradoiro beach is more sheltered; it is protected by a projection of the coastline and the swell conditions are softer. In the earliest samplings, closer to the *Prestige* spill, Lira mussels had higher TPAH levels because the oil reached this beach faster. Ancoradoiro mussels had higher TPAH contents in the latest samplings because the oil reached later and the softer swell cleaned the coast in a less effective manner.

In most samplings, mussel hydrocarbon content decreased during the recovery phase in the laboratory, due to the excretory activity of the mussels. Nevertheless, in reference mussels and in some samplings this content was

increased. In the reference mussels this might be caused by the presence in the water of PAH coming form small particles that could be adhered to the mussel shells. In the exposed mussels the most probable explanation lies in the external oil stains that mussels had in their shells. The intense aeration of the tanks might dissolve or disperse oil hydrocarbons in the seawater, and the mussels incorporated them by filtration.

Many recent studies on sea environment monitoring have used together mussels as sentinel organisms and comet assay as effect biomarker (Steinert et al., 1998a,b; Large et al., 2002; Rank and Jensen, 2003; Akcha et al., 2004; Haldörson et al., 2004), demonstrating the utility and efficiency of these organisms in evaluating the extent of a contamination event in the marine environment, and its possible repercussions on ecosystem and on human activities. Most of these works showed that gill cells result very suitable for comet assay evaluation, since they filter great quantities of contaminant agents present in water. Moreover, hydrocarbon exposure has been previously determined in mussels by means of comet assay (Steinert, 1996; Mitchelmore et al., 1998; Hamoutene et al., 2002; Pérez-Cadahía et al., 2004; Taban et al., 2004).

DNA damage in mussels from Lira and Ancoradoiro was significantly higher than in reference mussels, both before and after the recovery stage, but they were not significantly different from each other. The 7-day stay in the laboratory with clean fresh seawater significantly reduced comet tail length in mussels from the two locations exposed to *Prestige* oil, but did not alter results obtained in reference mussels. This was indicative of a certain DNA repair in exposed mussels, although it was not enough as to eliminate the significant difference with reference mussels, probably because the recovery time assayed was insufficient. Nevertheless, in some samplings tail length values were higher after the recovery phase, as a consequence of the external oil stains on the mussel shells.

The correlation coefficients obtained for the association between TPAH content in seawater and comet tail length in mussels from both exposed locations and in the total data indicated a good relationship environmental dose/effect. Nevertheless, it was surprising to obtain poor correlation coefficients for the associations between TPAH content in mussel tissues and comet tail length, since PAH accumulated in the organisms are, in principle, responsible for the genotoxic effects detected. However, it must be taken into account that DNA damage induced by PAH could not be produced immediately, and that a certain extension of it could already have been repaired in the moment of performing the comet assay for its evaluation. On the other hand, *Prestige* oil also contained several heavy metals that could contribute to the genotoxic damage detected.

Results obtained in this study have shown that the environmental impact caused by *Prestige* oil spill persisted at least until June 2004. Thus, this study showed up the importance of monitoring sea contamination events during an extended time, not only in evaluating the presence of the contaminants in the environment but also in determining their bioaccumulation and their effect on the exposed organisms.

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